A novel ω3-fatty acid desaturase involved in the biosynthesis of eicosapentaenoic acid

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Long-chain $n-3$ PUFAs (polyunsaturated fatty acids) such as EPA (eicosapentaenoic acid; 20:5$n-3$) have important therapeutic and nutritional benefits in humans. In plants, cyanobacteria and nematodes, ω3-desaturases catalyse the formation of these $n-3$ fatty acids from $n-6$ fatty acid precursors. Here we describe the isolation and characterization of a gene (*sdd17*) derived from an EPA-rich fungus, *Saprolegnia diclina*, that encodes a novel ω3-desaturase. This gene was isolated by PCR amplification of an *S. diclina* cDNA library using oligonucleotide primers corresponding to conserved regions of known ω3-desaturases. Expression of this gene in *Saccharomyces cerevisiae*, in the presence of various fatty acid substrates, revealed that the recombinant protein could exclusively desaturate 20-carbon $n-6$ fatty acid substrates with a distinct preference for ARA (arachidonic acid; 20:4$n-6$), converting it into EPA. This activity differs from that of the known ω3-desaturases from any organism. Plant and cyanobacterial ω3-desaturases exclusively desaturate 18-carbon $n-6$ PUFAs, and a *Caenorhabditis elegans* ω3-desaturase preferentially desaturated 18-carbon PUFAs over 20-carbon substrates, and could not convert ARA into EPA when expressed in yeast. The *sdd17*-encoded desaturase was also functional in transgenic somatic soya bean embryos, resulting in the production of EPA from exogenously supplied ARA, thus demonstrating its potential for use in the production of EPA in transgenic oilseed crops.

Key words: arachidonic acid, desaturase, eicosapentaenoic acid, fatty acid, *Saprolegnia*, transgenic oil.

INTRODUCTION

Long-chain PUFAs (polyunsaturated fatty acids) that contain 20 or 22 carbon atoms are essential components of membrane phospholipids, and many serve as precursors of eicosanoids such as prostaglandins, leukotrienes and thromboxanes. Two major classes of PUFA are the $n-6$ and $n-3$ fatty acids, and in mammals these are metabolically and functionally distinct. An excess of $n-6$ PUFAs such as ARA (arachidonic acid; 20:4$n-6$) shifts the physiological state to one that is prothrombotic and pre-aggregratory, leading to inflammatory and cardiovascular complications [1]. On the other hand, $n-3$ PUFAs such as EPA (eicosapentaenoic acid; 20:5$n-3$) have been shown to have therapeutic value in the prevention and treatment of numerous diseases, including cardiovascular disease [2], inflammation [3], arthritis [4] and cancer [5]. Thus an optimal balance of $n-6$ to $n-3$ PUFAs in the human diet is important for homoeostasis of inflammatory responses.

In mammals, long-chain PUFAs are synthesized by a group of membrane-bound desaturases and elongating enzymes [6,7]. Synthesis of these occurs through an alternating series of reactions starting with LA (linoleic acid; 18:2$n-6$) for the $n-6$ series, and ALA (α-linolenic acid; 18:3$n-3$) for the $n-3$ series (Scheme 1). ω3-desaturases catalyse the conversion of LA into ALA (Scheme 1), and since mammals lack both an ω3-desaturase and a Δ12-desaturase, LA and ALA are essential dietary components. Although the Western diet provides an adequate supply of $n-6$ PUFAs, the dietary intake of $n-3$ PUFAs is generally much lower. In light of their beneficial role, economical dietary sources of $n-3$ PUFAs such as EPA are being sought. EPA can be obtained from fish and algal oils, but these are not always economical or suitable for human use. Since plant oils are currently the largest source of $n-6$ PUFAs in the diet, a transgenic plant oil enriched in $n-3$ PUFAs such as EPA would be an attractive alternative to fish oil. Such an oil might be obtained by genetically manipulating the PUFA biosynthesis pathway in plants so as to produce long-chain $n-3$ PUFAs, such as EPA and docosahexaenoic acid. Hence there is a need to identify genes that encode enzymes involved in the biosynthesis of EPA.

ω3-desaturases are enzymes involved in the biosynthesis of $n-3$ PUFAs from $n-6$ fatty acids. All known plant and cyanobacterial ω3-desaturases can desaturate the 18-carbon PUFA substrate LA to generate ALA, but cannot act on 20-carbon PUFAs [8,9]. A *Caenorhabditis elegans* ω3-desaturase, FAT1, was reported to be more promiscuous in that it could desaturate 20-carbon PUFAs, in addition to 18-carbon PUFA substrates [10]. However, detailed enzymic characterization of FAT-1 later revealed that this enzyme predominantly desaturated 18-carbon PUFAs such as LA and ALA, with very low activity towards some 20-carbon PUFA substrates [9]. These studies also revealed that FAT-1 could not desaturate the 20-carbon PUFA, ARA, to produce EPA [9]. Thus an ω3-desaturase that is capable of converting ARA into EPA has as yet to be identified.

We set out to isolate a gene that encodes such an ω3-desaturase thought to be involved in EPA production, from an EPA-rich fungus, *Saprolegnia diclina*. This organism was predicted to possess a 20-carbon PUFA-specific ω3-desaturase, based on radiolabelling studies, which revealed the *in vivo* conversion of labelled ARA into EPA, but no conversion of labelled 18-carbon $n-6$ PUFAs into their corresponding $n-3$ products [11]. Here

Abbreviations used: PUFA, polyunsaturated fatty acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; ALA, α-linolenic acid; DGLA, dihomo-γ-linolenic acid; ER, endoplasmic reticulum.

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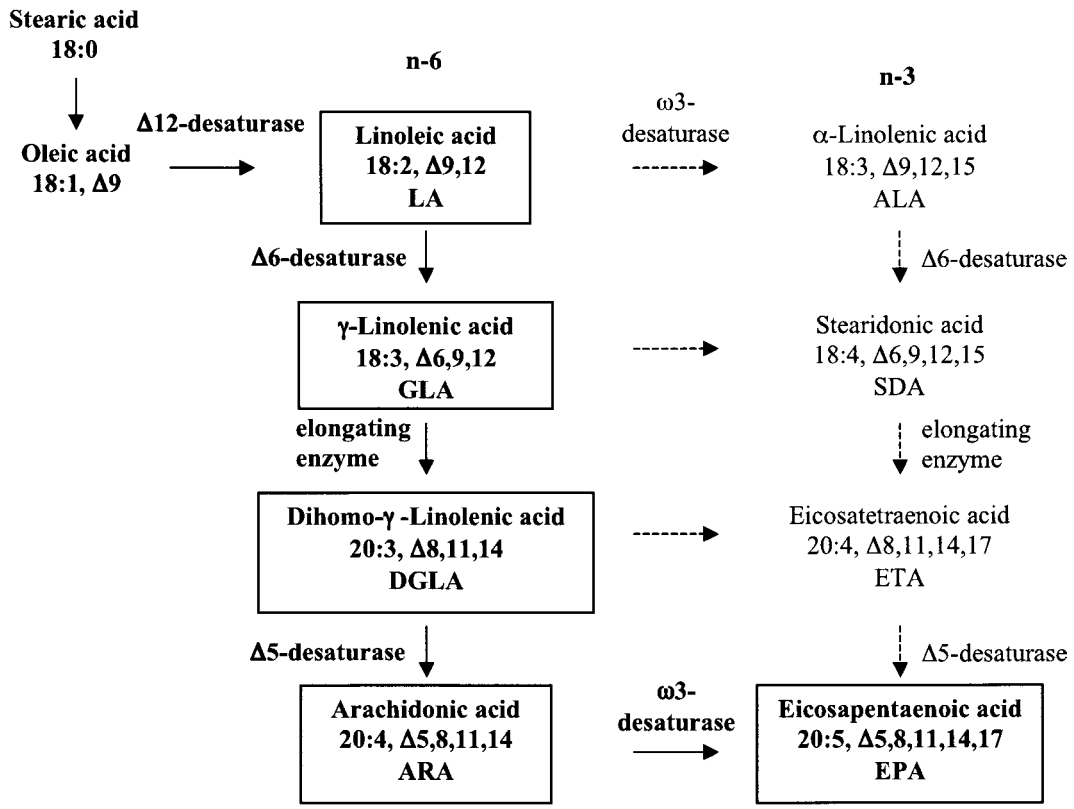
The nucleotide sequence reported in this paper has been submitted to the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AY373823.



Scheme 1 Biosynthesis pathway of long-chain PUFAs

Proposed metabolic pathway for EPA production in *Saprolegnia* (bold) based on PUFA intermediates detected in total lipid extracts (boxed).

we describe the isolation and characterization of a gene from *S. diclina*, designated *sdd17*, which encodes a unique ω3-desaturase with selectivity for 20-carbon PUFA substrates. Heterologous expression of this gene in bakers' yeast and somatic soya bean (*Glycine max*) embryos demonstrates its ability to convert ARA into EPA, with no activity on 18-carbon PUFA substrates. This is the first reported ω3-desaturase that does not utilize 18-carbon PUFA substrates, but can recognize and desaturate 20-carbon PUFA substrates, with a preference for converting ARA into EPA.

MATERIALS AND METHODS

Strains and growth conditions

S. diclina (ATCC 56851) cultures were grown in potato dextrose broth, Difco #336 (BD Diagnostic Systems, Sparks, MD, U.S.A.), at room temperature for 4 days with constant agitation at 250 rev./min. The mycelia were harvested by filtration through several layers of sterile cheesecloth, and frozen in liquid nitrogen for RNA extraction. The *Saccharomyces cerevisiae* strain used was SC334 (*matα pep4-3 prb1-1122 ura3-52 leu2-3, 112 reg1-501 gal1*) [12]. The *reg1-501* mutation alleviates glucose repression of the *GAL1* promoter in this strain. SC334 was grown either in YPD (yeast extract/peptone/dextrose) broth (Difco) or in selective media DOB (DropOut Broth; Qbiogene, Carlsbad, CA, U.S.A.) lacking leucine [DOB (−Leu)].

Construction of *S. diclina* cDNA library

Genomic DNA was isolated from *S. diclina* using the Plant Genomic DNA Extraction kit (Qiagen, Valencia, CA, U.S.A.) as per the manufacturer's protocol. To isolate total RNA, frozen cells were crushed in liquid nitrogen, incubated at 55 °C for 3 min in RLT buffer (Qiagen), and the lysate was homogenized using a Qiashredder column (Qiagen). The RNA was then isolated using the RNeasy Maxi kit (Qiagen) as per manufacturer's protocol. mRNA was isolated from total RNA using an oligo(dT)–cellulose resin, which was then used to synthesize double-stranded cDNA using the pBluescript II XR library construction kit (Stratagene, La Jolla, CA, U.S.A.). This cDNA was directionally cloned (5′ *Eco*RI, 3′ *Xho*I) into pBluescript II SK(+) vector (Stratagene) to generate the cDNA library, which contained $\approx 2.5 \times 10^6$ clones with an average insert size of $\approx$700 bp.

Cloning and expression of the *S. diclina* ω3-desaturase

Degenerate oligonucleotides (primers) were designed to contain conserved sequence motifs present in known ω3-desaturases. These included regions containing the three conserved histidine boxes found in most membrane-bound desaturases. PCR amplification was carried out in a 50 μl total volume containing 3 μl of the cDNA library template, PCR buffer [40 mM Tricine/KOH (pH 9.2), 15 mM potassium acetate, 3.5 mM magnesium acetate and 3.75 μg/ml BSA (final concentration)], 200 μM each dNTPs, primers (10 pmol each) and 0.5 μl of Advantage cDNA polymerase (BD Bioscience, Palo Alto, CA, U.S.A.). Of the various primer combinations tested, the only successful primer combination was RO1121 and RO1116. Primer RO1121 (forward; 5′-CCC TAC CAY GGC TGG CGC ATC TCG CAY CGC ACC CAY CAY CAG AAC-3′) corresponded to amino acid sequence PYHGWRISHRTHHQN, with the conserved histidine-box 2 motif (underlined), and primer RO1116 (reverse; 5′-GGT

GGC CTC GAY GAG RTG GTA RTG GGG GAT CTK GGG GAA GAR RTG-3′) corresponded to the protein motif H(L/F)FP-(Q/K)IPHYHL(V/I)EAT.

PCR amplification was as follows: initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 60 °C for 30 s and 72 °C for 1 min, and a final extension cycle of 72 °C for 7 min; the reaction was terminated at 4 °C. The 480 bp PCR fragment thus obtained was cloned into PCR-Blunt vector (Invitrogen, Carlsbad, CA, U.S.A.) and sequenced. The 5′ and 3′ ends of the gene were PCR-amplified from the cDNA library using primer RO1185 (5′-GGT AAA AGA TCT CGT CCT TGT CGA TGT TGC-3′) or primer RO1188 (5′-TAC GCG TAC CTC ACG TAC TCG CTC G-3′) in combination with the vector primers. Conditions for PCR amplification, using the *Taq* PCR Master Mix (Qiagen) along with 10 pmol of each primer and the cDNA library as a template, were identical with that described above. The resulting 5′- and 3′-end fragments of this putative ω3-desaturase were gel-purified, cloned and sequenced.

The full-length putative ω3-desaturase gene was PCR-amplified from the *S. diclina* cDNA library using the *Taq* PCR Master Mix and 10 pmol each of the following primers: RO1212 (5′-TCA ACA *GAA TTC* ATG ACC GAG GAT AAG ACG AAG GTC GAG TTC CCG-3′), containing the ATG start site (underlined) and an *Eco*RI cloning site (italic), and reverse primer RO1213 (5′-AAA AGA *AAG CTT* CGC TTC CTA GTC TTA GTC CGA CTT GGC CTT GGC-3′) containing the TAA stop codon (underlined) and a *Hin*dIII cloning site (italics). The resultant ≈1 kb PCR band was gel-purified, cloned into PCR-Blunt vector, and sequenced. This full-length 1077 bp gene was designated *sdd17*. The putative identity of the *sdd17*-encoded protein was assigned by sequence comparison with translated sequences in the NCBI public database using the TFasta program [13]. Multiple sequence alignments were carried out using AlignX, a modified ClustalW algorithm (InforMax, Bethesda, MD, U.S.A.). For construction of a phylogenetic tree, sequences were aligned using HMMalign and a hidden Markov model downloaded from the Pfam database (accession number PF00487) [14]. The alignment was optimized manually, and 215 amino acid positions at which the alignment was considered reliable (and that did not include a gap in any sequence) were selected for tree building. The tree was constructed by a protein distance matrix method using the *Protdist* and *Fitch* programs of the Phylip package [15]. A total of 100 bootstrap replicate data sets were used to test the reliability of the tree.

sdd17 was cloned into the *Eco*RI/*Hin*dIII sites of the yeast expression vector pYX242 (Novagen, Madison, WI, U.S.A.) to generate a construct labelled pRSP19, which was transformed into *S. cerevisiae* (SC334) using the Alkali-Cation Yeast Transformation kit (Qbiogene). Transformants were selected for leucine auxotrophy on DOB (−Leu) medium. To characterize enzyme activity, transformants were grown at 24 °C for 48 h in DOB (−Leu) medium containing 25–50 μM of various exogenously supplied fatty acid substrates, followed by whole-cell fatty acid analysis. The host strain transformed with vector alone was used as a negative control in all experiments.

Fatty acid analysis

Yeast cells expressing *sdd17* were washed in deionized water, vortex-mixed in 15 ml of methanol, and incubated for 1–2 h at room temperature in 29 ml of chloroform containing ≈100 μg of tridecanoin. Following the addition of 9 ml of distilled water, the lipids were extracted into the lower chloroform layer. The lipids were then filtered through a Whatman filter with 1 g of anhydrous Na_2SO_4, and the organic solvents were evaporated to dryness at 40 °C under a stream of nitrogen. The extracted lipids were saponified with 2 ml of 0.5 M KOH in methanol by heating at 95–100 °C for 30 min, and then cooled to room temperature. For fatty acid methylation, ≈2 ml of 14% BF_3 in methanol was added, the mixture heated to 95–100 °C for 30 min, and cooled to room temperature. Water (2 ml) was then added and fatty acid methyl esters were extracted with 1 ml of hexane. Fatty acid methyl esters were analysed by GLC as described previously [16]. The conversion rate of substrate into product (% conversion = 100 × [product]/[product + substrate]) was calculated to reflect the enzymic activity of the desaturase.

Total lipid extracts were fractionated into different lipid fractions (i.e. triacylglycerol, diacylglycerol, monoacylglycerol, non-esterified fatty acids and total phospholipids) by TLC using a solvent system of hexane/diethyl ether/acetic acid (70:30:1, by vol.). Each fraction was then scraped off and methylated with BF_3/methanol, and the quantity of fatty acid methyl esters measured by GLC.

Expression in somatic soya bean embryos

The vector pKS203 was used for transgenic expression of *sdd17* in somatic soya bean (*G. max*) embryos. This vector contained the coding sequence of *sdd17* that was obtained by PCR amplification using pRSP19 as template, flanked by the seed-specific promoter of the α′-subunit of β-conglycinin gene [17], and the phaseolin termination sequence [18]. The hygromycin B phosphotransferase gene [19] used for bacterial selection of this vector was under the control of the T7 RNA polymerase promoter. Plant selection of this vector was conferred by a second hygromycin B phosphotransferase gene under the control of the cauliflower mosaic virus 35 S promoter. pKS203 was transformed into soya bean embryos of cultivar Jack using the particle bombardment method [20], and selection, propagation and maturation of the transgenic somatic embryos were carried out as described previously [21]. Mature embryos were then washed and fatty acid methyl esters were extracted, separated and quantified as described previously [21].

RESULTS

Isolation of an ω3-desaturase gene from *S. diclina*

A PCR-based cloning strategy was adopted to identify the gene encoding a putative ω3-desaturase, using a directional cDNA library from *S. diclina*. The amino acid sequences of several known ω3-desaturases were aligned, conserved motifs identified, and degenerate oligonucleotides designed based on those motifs. Some of these conserved motifs included the three histidine boxes that are known to be required for desaturase enzymic activity [22]. Only one set of degenerate primers which contained the histidine box 2, and a region downstream of histidine box 3, was successful in the PCR amplification of a fragment with homology to known ω3-desaturases. This fragment was then used to isolate the full-length gene, *sdd17*, from the cDNA library. The open reading frame of *sdd17* was 1077 bp in length, and encoded a protein of 358 amino acids. Sequence analysis of the *sdd17*-encoded protein (SDD17) revealed that this protein shared greater similarity to ω3-desaturases than to Δ12-desaturases, although it displayed <45% amino acid identity with any reported ω3-desaturase (Figure 1). Alignment of SDD17 with the *C. elegans* ω3-desaturase [10] indicated that the two proteins shared only 31.6% sequence identity with each other (Figure 1). Phylogenetic comparison of SDD17 with representatives from the ω3- and Δ12-desaturase family revealed that SDD17 did not cluster with any of the known ω3- or Δ12-desaturases (Figure 2). SDD17 did contain

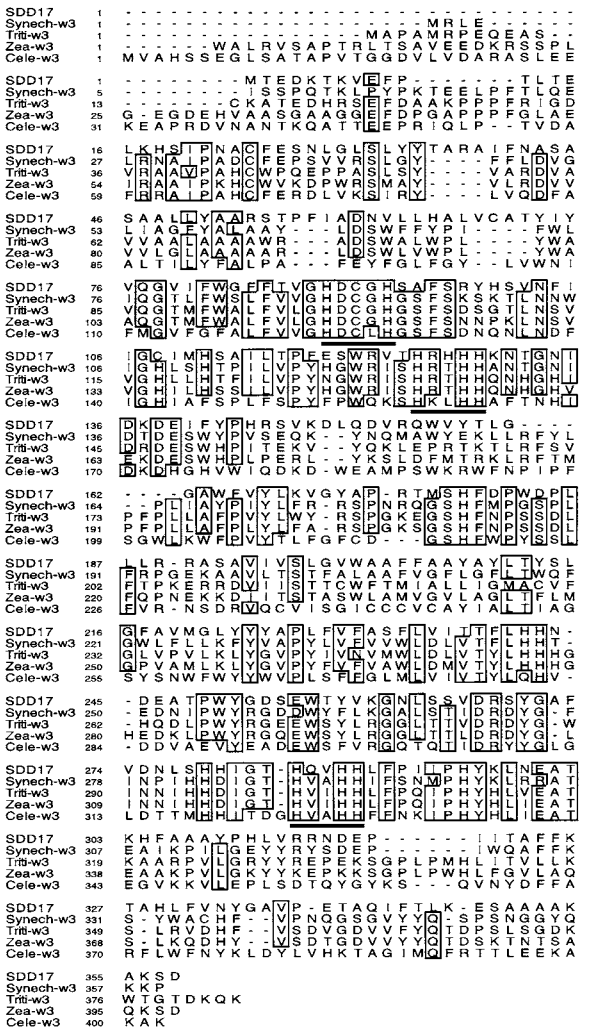


Figure 1 Amino acid sequence comparison of the ω3-desaturase from *S. diclina* (SDD17) with other ω3-desaturases

Identical residues are boxed. The conserved histidine boxes are underlined. The alignment includes amino acid sequences from *Synechocystis* sp. PCC6803 (Synech-w3; S52650), *Triticum aestivum* (Triti-w3; BAA28358), *Zea mays* (Zea-w3; BAA22440) and *Caenorhabditis elegans* (Cele-w3; Q21056).

the three conserved histidine-box motifs found to be present in all membrane-bound desaturases (Figure 1).

Functional characterization of SDD17 in yeast

To determine the functionality of SDD17, yeast was transformed with pRSP19, a construct consisting of *sdd17* cloned into the yeast expression vector pYX242. The recombinant yeast was grown in the presence of various PUFA substrates in selective media, and the new fatty acids formed in the lipid fraction of the yeast were analysed by GLC. To check for ω3-desaturase activity on 18-carbon PUFAs, the substrates LA or γ-linolenic acid (18:3$n-6$) were added to the cultures individually, and monitored for conversion into ALA or stearidonic acid (18:4$n-3$), respectively. SDD17 did not desaturate any of these 18-carbon PUFA substrates (Table 1, Figure 3). However, when 20-carbon PUFA substrates such as ARA or DGLA (dihomo-γ-linolenic acid; 20:3$n-6$) were added to the cultures, EPA or eicosatetraenoic acid (20:4$n-3$) were produced respectively, indicating that SDD17 possessed ω3-desaturase activity specific for 20-carbon PUFAs (Table 1, Figure 3). SDD17 was also tested for Δ6- and Δ5-desaturase activity by monitoring for conversion of LA into γ-linolenic acid, or DGLA into ARA respectively, but none was detected (results not shown). In addition, SDD17 could not convert endogenous oleic acid (18:1$n-9$) into LA in the recombinant yeast culture, indicating that *sdd17* did not encode a Δ12-desaturase.

The results in Table 1 indicate that ARA was the preferred substrate for SDD17, 25% of which was converted into EPA. The gas chromatogram in Figure 3 shows the accumulation of a new fatty acid peak in the lipid fraction of the recombinant yeast expressing *sdd17*, when compared with the control, when incubated in presence of ARA. This new peak had a retention time identical with that of authentic EPA, and GLC-MS analysis indicated that the new fatty acid peak had a mass peak at $m/z = 287$, and a fragmentation pattern identical with that of EPA (results not shown). These findings confirm that SDD17 can convert ARA into EPA.

Fractionation of the total lipids of yeast expressing *sdd17* revealed that almost all of the exogenously supplied ARA that was taken up by the cells remained as a non-esterified conjugated form (possibly linked to CoA), and was not incorporated into the membrane phospholipids (results not shown). The EPA subsequently produced was also localized in the non-esterified fatty acid fraction and was not detected in the phospholipid fraction. This suggests that in yeast, SDD17 can desaturate non-phospholipid-linked substrates.

Expression of *S. diclina* ω3-desaturase in somatic soya bean embryos

To verify its ability to function as an ω3-desaturase in higher plants, *sdd17* was expressed in somatic soya bean embryos. Although soya beans cannot synthesize PUFAs longer than LA or ALA, the embryos can take up small amounts of exogenous PUFA substrates, which can then be utilized as substrates to test for desaturase activity. When transgenic embryos expressing *sdd17* were incubated in the presence of exogenous ARA, the embryos were able to take in ARA and convert ≈50% of it into EPA (Table 2). EPA was not detected in untransformed embryos, or in embryos containing vector alone (Table 2). In addition, in the transgenic embryos expressing *sdd17*, the levels of endogenous ALA did not increase above that seen in the controls despite the availability of large amounts of LA substrate. This confirms the conclusions reached from the yeast expression studies that the *sdd17*-encoded ω3-desaturase does not recognize and desaturate 18-carbon PUFA substrates such as LA.

DISCUSSION

In the EPA-rich fungus *Saprolegnia*, the $n-6$ PUFA pathway is predominant, and EPA is the sole $n-3$ PUFA detected in the total lipids [11,23] (Scheme 1). This observation, in addition to results from radiolabelling studies [11], led to the prediction that a novel ω3-desaturase must exist in *Saprolegnia* that converted ARA into EPA, but was unable to recognize 18-carbon PUFA substrates. The *sdd17* gene described here encodes an ω3-desaturase with these properties. Expression of *sdd17* in yeast revealed that the encoded protein could exclusively desaturate 20-carbon PUFAs, with a preference for ARA. In these cells, the levels of EPA reached ≈6% by weight of total lipid. It is possible that higher EPA levels can be achieved if *sdd17* were to be expressed in an oleaginous organism that can store EPA in the triacylglycerol form.

The feasibility of utilizing *sdd17* in an oil-seed crop for production of EPA was demonstrated by expressing it in somatic soya bean embryos. Somatic soya bean embryos are similar to

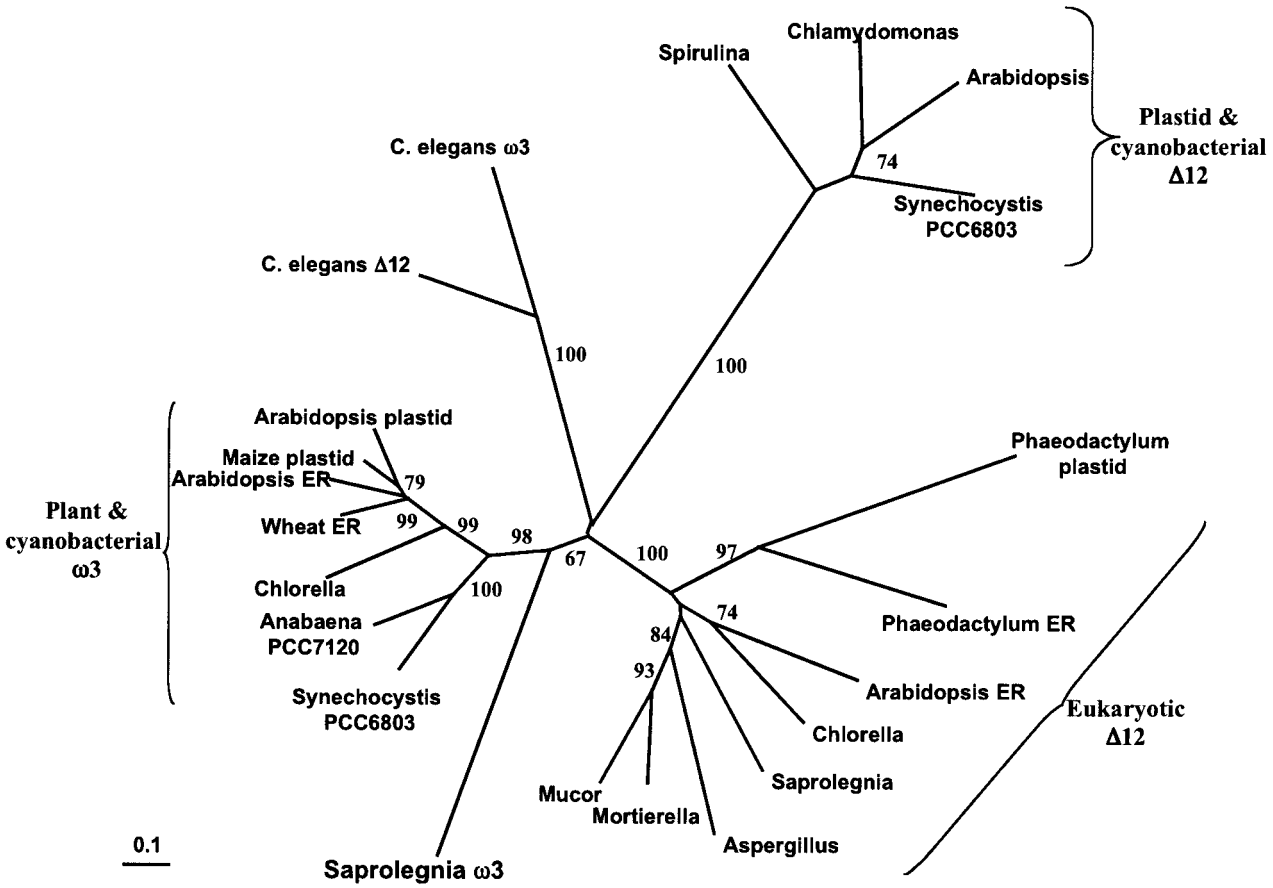


Figure 2 Phylogenetic tree of ω3- and Δ12-desaturases

This protein distance-matrix tree shows the evolutionary relationships between ω3- and Δ12-desaturases from eukaryotes and cyanobacteria. Bootstrap values > 65 are shown adjacent to branches. The scale bar represents 0.1 mutational changes per residue [10 PAM (percent accepted mutations) units]. ω3-desaturases: *Anabaena* sp. PCC7120 (Q8YWL1), *Arabidopsis thaliana* plastid (P46310), *A. thaliana* ER (P48623), *Caenorhabditis elegans* (Q21056), *Chlorella vulgaris* (Q8W3L3), *Synechocystis* sp. PCC6803 (Q55240), *Triticum aestivum* ER (wheat; O65792), *Zea mays* plastid (maize; O24626). Δ12 desaturases: *A. thaliana* plastid (P46312), *A. thaliana* ER (P46313), *Aspergillus nidulans* (Q9HF05), *C. elegans* (Q9XUB8), *Chlamydomonas* sp. (Q9SBU4), *C. vulgaris* (Q8W3L4), *Mortierella alpina* (Q9Y8H5), *Mucor circinelloides* (Q96VC2), *Phaeodactylum tricornutum* plastid (AY165024), *Phaeodactylum tricornutum* ER (AY165023), *S. diclina* (AY373822), *Spirulina platensis* (Q54794), *Synechocystis* sp. PCC6803 (P20388).

Table 1 Production of EPA in recombinant yeast expressing the *S. diclina* ω3-desaturase gene *sdd17* (pRSP19)

Substrate (%) and product (%) both refer to the percentage (w/w) of total fatty acid. % conversion = 100 × ([product]/[product + substrate]).

Substrate	Substrate (%)	Product	Product (%)	% Conversion
pRSP19				
18:2$n-6$*	9.13 ± 0.29	18:3$n-3$	0	0
18:3$n-6$*	9.92 ± 0.81	18:4$n-3$	0	0
20:3$n-6$†	20.07 ± 0.28	20:4$n-3$	1.05 ± 0.06	4.98
20:4$n-6$†	17.82 ± 0.82	20:5$n-3$	6.25 ± 0.42	25.9
pYX242				
18:2$n-6$*	10.07 ± 0.81	18:3$n-3$	0	0
18:3$n-6$*	10.23 ± 0.16	18:4$n-3$	0	0
20:3$n-6$†	7.58 ± 2.09	20:4$n-3$	0	0
20:4$n-6$†	9.15 ± 1.48	20:5$n-3$	0	0

* 50 μM exogenous substrate added.
† 25 μM exogenous substrate added.

soya bean seeds in that they are rich in triacylglycerols, and their fatty acid composition is predictive of that of the seeds derived from such embryos [24]. Results from this preliminary study demonstrated that SDD17 was active in these embryos, converting almost half of the ARA substrate that was taken up by the embryo to EPA. Although the uptake of ARA by the embryos was low, the presence of EPA in soya bean embryos expressing *sdd17*, and its absence in the controls, was evidence of SDD17 activity.

SDD17 shares all the conserved features present in other membrane-bound acyl-lipid desaturases. These include the presence of two long stretches of hydrophobic residues that traverse the lipid bilayer, and three histidine-rich motifs proposed to be involved in the ligation of iron atoms within the active-site domain of these enzymes [22]. This protein also contains the C-terminal motif, KAKSD, proposed to be a retention signal for many transmembrane proteins in the ER (endoplasmic reticulum) [25]. Like other ω3-desaturases, this protein does not contain a fused cytochrome b_5 domain at its N-terminus and is thus assumed to interact with a separate cytochrome b_5 for its activity. However, unlike plant ω3-desaturases that are thought to desaturate predominantly phospholipid-linked substrates, SDD17 is capable of desaturating non-esterified (CoA-linked) ARA in yeast. It is possible that this enzyme can desaturate phospholipid-linked substrates as well. However, this could not be established in yeast, since there was no incorporation of the ARA substrate into the yeast membrane phospholipids.

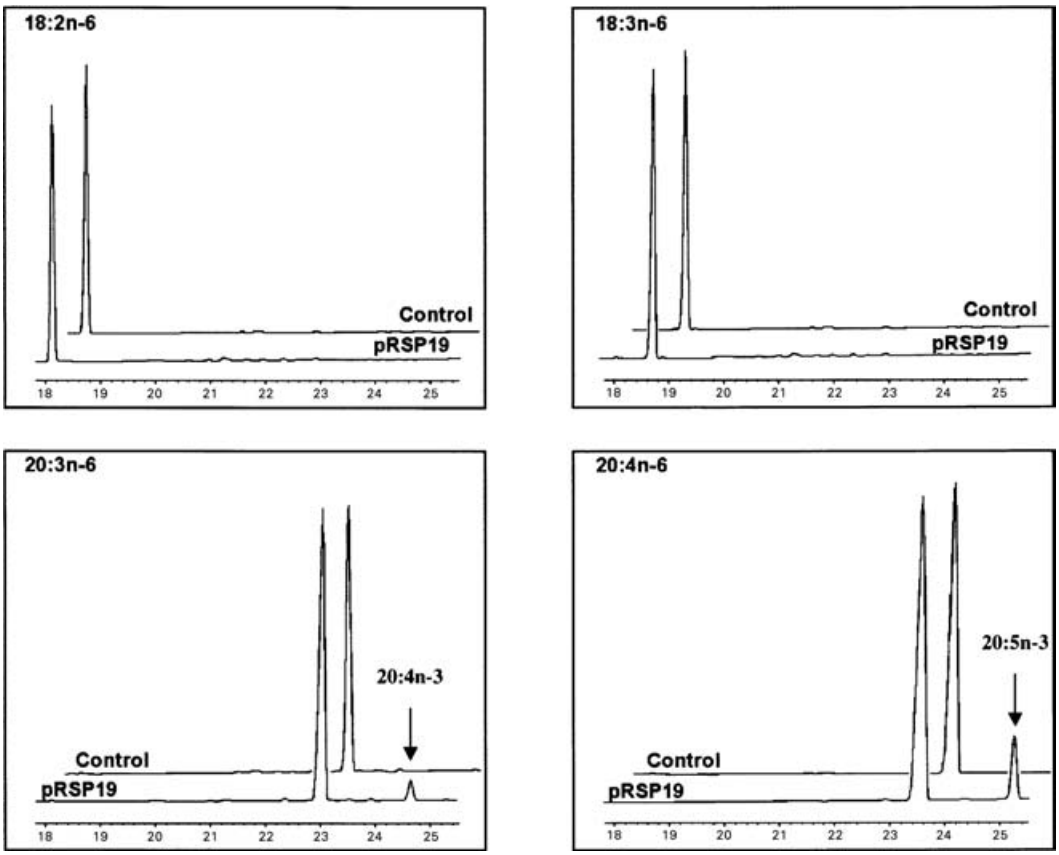


Figure 3 Gas chromatogram of fatty acid methyl esters from lipid fraction of yeast containing pRSP19 or pYX242

All yeast strains were grown in minimal media supplemented with exogenous LA (18:2$n-6$), γ-linolenic acid (18:3$n-6$), DGLA (20:3$n-6$) and ARA (20:4$n-6$).

Table 2 Fatty acid composition of somatic soya bean embryos from an untransformed line, and transgenic lines incubated in the presence of ARA that contained vector alone (+ Vector), or the *S. diclina* ω3-desaturase gene *sdd17* (+ *sdd17*)

Measurements are presented as a weight percentage of total fatty acids of embryos and were obtained from independent analysis of five different embryos (mean ± S.D.). ND, not detected.

	Total fatty acid (weight %)		
Fatty acid	Untransformed	+ Vector	+ *sdd17*
18:0	2.0 ± 0.1	3.5 ± 0.5	3.4 ± 0.6
18:1	9.4 ± 1.2	10.9 ± 1.8	7.3 ± 1.5
18:2$n-6$	53.5 ± 1.2	49.9 ± 5.0	50.8 ± 2.2
18:3$n-3$	18.5 ± 1.9	17.1 ± 2.4	17.9 ± 2.2
20:4$n-6$	ND	3.7 ± 0.8	1.3 ± 0.4
20:5$n-3$	ND	ND	0.9 ± 0.2

Phylogenetic analysis (Figure 2) shows that SDD17 is not specifically related to any of the previously identified ω3- or Δ12-desaturases, including its own Δ12-desaturase previously identified in our laboratory (S. L. Pereira, unpublished work). The tree illustrates a number of evolutionary patterns: fungi, plants and algae have an ER-localized Δ12-desaturase. Higher plants and green algae have a second Δ12-desaturase that is localized to the plastid with an apparent cyanobacterial evolutionary origin. In the case of the ω3-desaturases, both the plastidal- and the ER-localized ω3-desaturase from higher plants appear to have evolved from ancestral cyanobacterial enzymes, suggesting that duplicated genes acquired different targeting signals either as part of or following the process of gene transfer from the chloroplast to the nucleus. This phylogenetic tree reveals that the ω3- and Δ12-desaturases from *C. elegans* and *S. diclina* have evolved separately in the two organisms. In *C. elegans*, the ω3- and Δ12-desaturases are closely related, suggesting a recent evolutionary origin. This is not the case in *S. diclina*; here the Δ12-desaturase is typical of the known fungal and plant ER-localized Δ12-desaturases, but the ω3-desaturase is quite distinct from all the other enzymes.

The ω3-desaturases from *C. elegans* (FAT-1) and *S. diclina* (SDD17) have rather different substrate specificities. FAT-1 shares similar substrate specificity with the ω3-desaturases from plants, recognizing 18-carbon $n-6$ PUFAs as its preferred substrates [9]. Although FAT-1 can recognize the 20-carbon substrate DGLA, the enzymic activity towards DGLA is significantly lower than activity towards the 18-carbon $n-6$ PUFAs [9]. FAT-1 also has no detectable activity on ARA [9]. In contrast, SDD17 does not recognize 18-carbon PUFAs, but preferentially desaturates the 20-carbon PUFA, ARA. It is possible that the pathway for *in vivo* biosynthesis of EPA in *S. diclina* and *C. elegans* is quite different. In *Saprolegnia*, studies have predicted the direct involvement of a SDD17-like desaturase in the formation of EPA from ARA [11,23]. With the exception of EPA, no other $n-3$ PUFAs are detected in the lipids of *Saprolegnia* [11,23], suggesting that EPA is directly synthesized from ARA *in vivo*. In contrast, analysis of the lipids of *C. elegans* revealed the presence of $n-3$ PUFA intermediates such as ALA and eicosatetraenoic acid (20:4$n-3$) [26], indicating that EPA may be produced via the $n-3$ PUFA pathway (Scheme 1) without the involvement of ARA as a

substrate. Here FAT-1 may function in the conversion of 18-carbon $n-6$ PUFAs into their corresponding $n-3$ products, which are then used for EPA biosynthesis.

The identification of SDD17 expands the functional repertoire of the ω3-desaturase family because of its novel substrate recognition property. Due to lack of a crystal structure, progress has been slow in structurally characterizing these hydrophobic membrane-bound desaturases. However, structure–function predictions are possible, based on comparative sequence analysis of functionally related desaturases, which can then be tested by genetically manipulating these enzymes [22,27–29]. SDD17 may thus provide useful comparative information for elucidating the mechanism of substrate specificity among these desaturases.

The health benefits associated with the consumption of $n-3$ PUFAs such as EPA have resulted in an increased demand for renewable and economical sources of this PUFA. An EPA-enriched plant oil is one such candidate that may be obtained by transgenic expression of genes that encode enzymes involved in EPA biosynthesis. Studies with enzymes such as the Δ6- and Δ5-desaturases, and elongating enzymes (elongases) that are involved in EPA production (Scheme 1) have revealed that the same enzymes function in the biosynthesis of both $n-6$ and $n-3$ PUFAs [16,30,31]. Thus simultaneous transgenic expression of these genes would result in oils containing $n-6$ PUFAs such as ARA in addition to EPA, due to high endogenous levels of the $n-6$ precursor LA present in oilseed crops. Since ARA is a precursor for synthesis of proinflammatory eicosanoids involved in inflammatory and cardiovascular disease, its presence in oils targeted for adult nutrition is undesirable. Hence there is a need to develop strategies to maximize EPA yields while minimizing ARA levels in transgenic oils. The recent identification of a Δ6-desaturase with greater preference for ALA over LA [32] provides a means for increasing EPA yields by increasing the shunt through the $n-3$ PUFA pathway. The *S. diclina* ω3-desaturase, by virtue of its ability to convert ARA into EPA, provides a very useful tool for the removal of ARA from such EPA-enriched transgenic oils. Thus *sdd17* has applications for the production of designer oils in transgenic plants that may help satisfy the demands of the nutraceutical and pharmaceutical industries.

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